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Are Retinal Microvascular Phenotypes Associated With the *1675G/A* Polymorphism in the Angiotensin II Type-2 Receptor Gene?

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BACKGROUND
The X-linked angiotensin II type-2 receptor (*AT2R*) gene *1675G/A* polymorphism is located in the short intron 1 of the *AT2R* gene within a sequence motif conforming to a splice branch site. AT2R is expressed in the human retina, but no previous study examined the association between retinal microvascular phenotypes and the *AT2R 1675G/A* polymorphism.

METHODS
In 340 subjects randomly selected from a Flemish population (mean age, 51.9 years; 51.5% women), we post-processed retinal images using IVAN software to generate the retinal arteriole and venule equivalents (central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE)) and the arteriole-to-venule ratio (AVR). DNA fragments including the *AT2R 1675G/A* polymorphism were amplified by PCR. We applied a mixed model to assess phenotype–genotype associations while accounting for relatedness and covariables.

RESULTS
CRAE, CRVE, and AVR averaged 151.9 µm, 215.2 µm, and 0.710, respectively. CRAE was 5.5 µm greater in women than men and decreased with age ($P < 0.05$). In multivariable-adjusted analyses, CRAE was higher in hemizygous and homozygous carriers of the *AT2R A* allele than in their *G* allele counterparts in both sexes combined (+4.49 µm; $P = 0.014$) and in men (+4.91 µm; $P = 0.032$) with a similar trend in women (+3.41 µm; $P = 0.14$). AVR was increased in the presence of the *AT2R A* allele compared with *AT2R G* hemizygotes and homozygotes (+0.024; $P = 0.0082$). The associations of CRAE and CRVE with other polymorphisms were not significant.

CONCLUSIONS
Pending confirmation in experimental and epidemiological studies, our findings suggest that diameter of the retinal arterioles might be associated with the *AT2R 1675G/A* polymorphism.

Keywords: *angiotensin II type-2 receptor gene; blood pressure; hypertension; polymorphism; microcirculation; retina*

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The human angiotensin II type-2 receptor gene (*AT2R*) consists of three exons interspaced by two introns.[1] Regulatory elements are located in the promoter area and in the first intron, whereas the third exon contains the complete protein-coding sequence, which encode the 363-amino acid G-protein-coupled receptor.[1] *AT2R* contains several polymorphic sites. A common *G/A* polymorphism occurs at position 1675 (rs1403543) relative to the transcription start.[2] It is also known as *–1332G/A* relative to the start codon in the first intron.[2]

Fetal tissues express AT2R at high levels.[3] In adults, few organs express AT2R, but these tissues include the heart, kidney, and vascular endothelium.[4] *AT2R* is an X-chromosome linked gene.[5] In the European Project on Genes in Hypertension (EPOGH), we previously reported association between left ventricular structure and the *AT2R 1675G/A* polymorphism, which was modulated by sodium intake.[6] Arterial structure and function shows substantial differences between women and men. We hypothesized that variation in X-chromosome linked genes might contribute to these sex differences. An extensive literature search, using as key words "AT2R" or "angiotensin II type 2 receptor" or "angiotensin II receptor, type 2," did not reveal any previous publication on the association between vascular traits and genetic variants of *AT2R* in humans. We therefore analyzed the database of the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) for possible association between

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retinal microvascular phenotypes and the *AT2R 1675G/A* polymorphism.

METHODS

Study population. Previous publications describe the recruitment of FLEMENGHO participants in detail.[7] From August 1985 until November 1990, a random sample of the households living in a geographically defined area of northern Belgium was recruited with the goal to enroll an equal number of participants in each of six subgroups by sex and age (20–39, 40–59, and ≥60 years). All household members with a minimum age of 20 years were invited to take part, provided that the quota of their sex-age group had not yet been fulfilled. From June 1996 until January 2004 recruitment of families continued using the former participants (1985–1990) as index persons and also including subjects younger than 20 years. The participation rate at enrolment was 65.0%.[7]

From 10 January 2008 until December 2010, we reinvited former participants for a follow-up examination at our field centre, including imaging of the retinal microvessels. We obtained informed written consent from 418 participants (participation rate 77.8%). We removed 78 participants from analysis, because of low-quality retinal images (n = 54), missing genotypes (n = 20), or outlying data points, which were >3 s.d.s. away from the mean (n = 4). Thus, our analyses included 340 subjects.

Clinical and biochemical measurements. For at least 3 h before the vascular examinations, the participants refrained from heavy exercise, smoking, and intake of alcohol, and caffeine-containing beverages. Trained nurses measured the subjects' anthropometric characteristics and blood pressure. They administered a questionnaire to collect information about each participant's recent medical history, smoking and drinking habits, and intake of medications. Each subject's blood pressure was the average of five consecutive readings measured after the participants had rested in the sitting position for at least 5 min. Hypertension was defined as blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic, and/or use of antihypertensive drugs. Mean arterial pressure was diastolic blood pressure plus one third of the difference between systolic and diastolic blood pressure. Body mass index was weight in kilograms divided by the square of height in meters. On the day of the microvascular measurements, with the subjects fasting for at least 8 h, venous blood samples were drawn. Using standardized automated methods, we measured plasma glucose and insulin and total and high-density lipoprotein-cholesterol on serum. We computed the homeostasis model assessment of insulin resistance ($HOMA_{IR}$) as fasting serum insulin × fasting plasma glucose/22.5. Diabetes mellitus was a self-reported diagnosis, use of antidiabetic medication, or a fasting or casual blood glucose concentration ≥6.99 mmol/l (126 mg/dl) or ≥11.1 mmol/l (200 mg/dl), respectively.

Microvascular phenotyping. We phenotyped retinal arterioles and venules as described before.[8] To dilate the pupil, retinal imaging took place in a dimly lit examination room, after the subjects had accommodated to darkness for at least 5 min. Trained observers made photographs of each eye by means of a Canon Cr-DGi nonmydriatic retinal visualization system combined with a Canon D-50 digital camera (Canon, Kyoto, Japan). After converting JPEG to TIFF images, using Phatch (http://photobatch.stani.be/), two trained observers identified individual arterioles and venules for each image, using a validated computer-assisted program IVAN (Vasculo-matic ala Nicola, version 1.1; Department of Ophthalmology and Visual Science, University of Wisconsin-Madison, Madison, WI).[9] This software combines the individual measurements into summary indexes: the central retinal arteriolar equivalent (CRAE) and the central retinal venular equivalent (CRVE), based on formulas by Parr and Spears[10] and by Hubbard *et al.*[11] and Knudtson *et al.*[12] The arteriole-to-venule ratio (AVR) was CRAE divided by CRVE.

Genotyping. Genomic DNA was extracted from peripheral blood. DNA fragments that contained the polymorphism of AT2R (*AT2R* rs1403543, *1675G/A*) were amplified by PCR with forward 5′-ATTACGTCCCAGCGTCTGAG-3′ and reverse 5′-GGCACTAAGCAAGCTGATTTAT-3′ primers. The PCR products were digested by the addition of 5 U of *Hpy*188III restriction enzyme. In the presence of the *1675G* allele, the PCR product (255 bp) was cut into two fragments of 194 bp and 61 bp in length and visualized on ethidium bromide-stained 1.5% agarose gels.

Statistical methods. For database management and statistical analysis, we used SAS software (SAS Institute, Cary, NC), version 9.2. We normalized the distributions of insulin and $HOMA_{IR}$. We compared means and proportions, using Student's t-test and χ^2-test, respectively. Our statistical methods also included single and multiple linear regressions. We searched for possible covariables of the arterial phenotypes, using a stepwise regression procedure with the P values for independent variables to enter and to stay in the model set at 0.15. As covariables, we considered sex, age, body mass index, mean arterial pressure, high-density lipoprotein-to-total cholesterol, $HOMA_{IR}$, and design variables (0, 1), coding for current smoking, alcohol intake and use of various classes of antihypertensive drugs (diuretics, β-blockers, calcium channel blockers, and inhibitors of the renin–angiotensin–aldosterone system). We applied a generalization of the standard linear model, as implemented in the PROC MIXED procedure of SAS package, to investigate the associations between the retinal phenotypes and explanatory variables, while accounting for relatedness among study participants and adjusting for covariables.

RESULTS

Characteristics of the participants

Our study sample included 340 subjects from 157 families with a size ranging from 1 to 36 individuals. The participants included 175 women (51.5%) and 157 hypertensive patients (46.2%). Mean age was 51.9 years (range: 16.7–79.1). **Table 1** lists the characteristics of the participants by sex. Compared with men, women had lower blood pressures and plasma

glucose, but higher high-density lipoprotein-cholesterol. Of 340 participants, 64 (18.8%) were current smokers, and 210 (61.8%) reported regular alcohol intake. In current smokers of either sex, median tobacco use was 12 cigarettes/day (interquartile range: 7–20). In participants reporting alcohol intake, median alcohol intake was 5 g/day (interquartile range: 5–10) among women and 10 g/day (interquartile range: 6–12) among men. Only 2 participants (0.6%) had diabetes mellitus.

Retinal microvascular phenotypes

In all participants, CRAE, CRVE, and AVR averaged 151.9 µm, 215.2 µm, and 0.710, respectively (Table 2). CRAE and AVR were 5.5 µm and 0.015 higher in women than men, whereas CRVE was similar in both sexes (Table 2). CRAE and CRVE decreased with age (P for trend ≤0.0423) in women as well as men (Figure 1). In women, the correlation coefficients of CRAE and CRVE with age were –0.12 (P = 0.14) and –0.21 (P = 0.046), whereas in men these correlations were –0.24 (P = 0.0034) and –0.33 (P = 0.0019), respectively.

In stepwise regression analysis (Table 3), the covariables considered for entry into the model were sex, age, body mass index, mean arterial pressure, high-density lipoprotein-to-total cholesterol ratio, $HOMA_{IR}$, smoking, and drinking alcohol. Sex and age were forced into the model. In addition to sex and age, mean

Table 1 | Characteristics of participants

Characteristic	Women	Men	All
Number	175	165	340
Mean (s.d.) of characteristic			
Age (years)	50.8 ± 14.0	53.1 ± 14.8	51.9 ± 14.4
Body mass index (kg/m^2)	26.6 ± 4.9	27.1 ± 4.0	26.9 ± 4.5
Systolic blood pressure (mm Hg)	127.5 ± 17.5	133.5 ± 15.0***	130.4 ± 16.6
Diastolic blood pressure (mm Hg)	78.1 ± 9.6	83.0 ± 10.1***	80.5 ± 10.1
Mean arterial pressure (mm Hg)	94.6 ± 10.6	99.8 ± 10.5***	97.1 ± 10.9
Total cholesterol (mmol/l)	5.26 ± 0.89	5.26 ± 1.06	5.26 ± 0.970
HDL-lipoprotein (mmol/l)	1.58 ± 0.35	1.31 ± 0.28***	1.45 ± 0.34
HDL-to-total cholesterol	0.30 ± 0.07	0.26 ± 0.06	0.28 ± 0.07
Fasting blood glucose (mmol/l)	4.89 ± 0.58	5.20 ± 1.10**	5.04 ± 0.89
Geometric mean (95% CI) of characteristic			
Fasting insulin (mU/l)	5.13 (2.00–14.00)	5.20 (2.00–15.00)	5.16 (2.00–14.50)
$HOMA_{IR}$	1.11 (0.42–3.95)	1.18 (0.41–3.81)	1.14 (0.41–3.88)
Number of subjects (%) with characteristic			
Current smoking	37 (21.1)	27 (16.5)	64 (18.8)
Drinking alcohol	86 (49.1)	124 (75.1)***	210 (61.8)
Hypertension	71 (40.6)	86 (52.1)*	157 (46.2)
Antihypertensive drug treatment			
Diuretics	30 (17.1)	21 (12.7)	51 (15.0)
β-Blockers	24 (13.7)	35 (21.2)	59 (17.3)
Vasodilators	10 (5.7)	12 (7.3)	22 (6.5)
RAAS inhibitors	14 (8.0)	22 (13.3)	36 (10.6)

Hypertension was a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic, and/or use of antihypertensive drugs. Using of vasodilators included calcium channel blockers and α-blockers. Renin–angiotensin–aldosterone system inhibitors included angiotensin II converting enzyme inhibitors and angiotensin II type 1 receptor blockers.
CI, confidence interval; HDL, high-density lipoprotein; $HOMA_{IR}$, homeostasis model assessment of insulin resistance; RAAS, renin–angiotensin–aldosterone system.
Significance of the difference between men and women: *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2 | Retinal vascular phenotypes of participants

Characteristic	Women	Men	All
Number	175	165	340
Central retinal arteriolar equivalent (µm)	154.6 ± 14.7	149.1 ± 15.2***	151.9 ± 15.2
Central retinal venular equivalent (µm)	216.6 ± 20.0	213.8 ± 20.1	215.2 ± 20.1
Arteriole-to-venule ratio	0.717 ± 0.065	0.702 ± 0.071*	0.710 ± 0.068

Values were mean ± s.d.
Significance of the difference between women and men: *P < 0.05; ***P < 0.001.

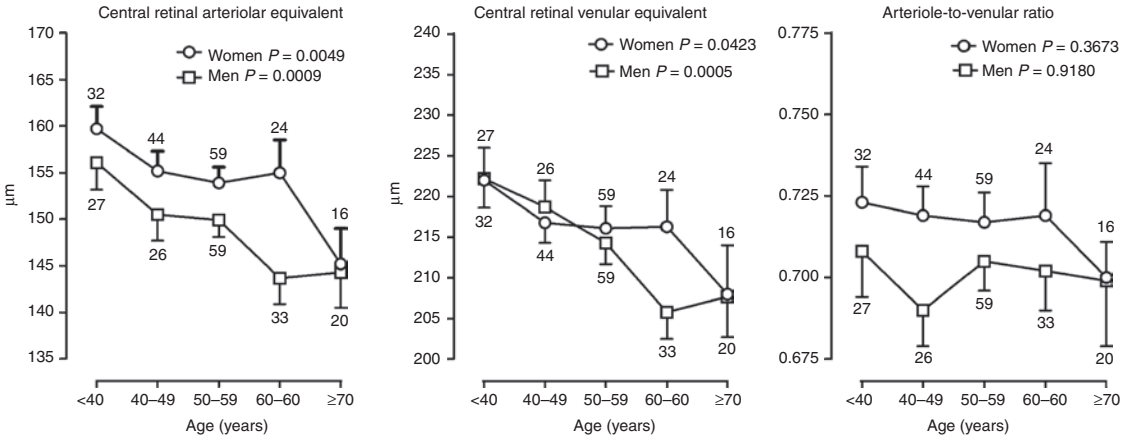

Figure 1 | Retinal vascular phenotypes by sex and age. Data are mean ± s.e. *P* values are for linear trend across the age groups.

Table 3 | Determinants of retinal vascular phenotypes

Determinant	Central retinal arteriolar equivalent	Central retinal venular equivalent	Arteriole-to-venule ratio
R^2	0.142	0.073	0.037
Intercept	1.461	0.449	–0.412
Regression coefficients			
Sex (0, 1)	3.585 ± 1.583*	1.852 ± 2.121	0.007 ± 0.008
Age (years)	–0.190 ± 0.056***	–0.289 ± 0.074***	–0.816 ± 2.713 (×10^4)
Mean arterial pressure (mm Hg)	–0.237 ± 0.078***	—	–0.829 ± 0.338 (×10^3)*
Current smoking (0, 1)	5.763 ± 2.000***	7.723 ± 2.706**	—

The covariables considered for entry into the model were sex, age, BMI, mean arterial pressure, HDL-to-total cholesterol, $HOMA_{IR}$, current smoking, and drinking alcohol. Sex and age were forced into the models.
BMI, body mass index; HDL, high-density lipoprotein; $HOMA_{IR}$, homeostasis model assessment of insulin resistance.
Significance of the multiple partial correlation: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4 | Allele and genotype frequencies

Gene	Allele		Genotype			*P*
AGTR2 (women)	A	G	AA	AG	GG	
	186 (53.1)	164 (46.9)	50 (28.6)	86 (49.1)	39 (22.3)	0.99
AGTR2 (men)	A	G	A		G	
	86 (52.1)	49 (47.9)	86 (52.1)		49 (47.9)	—

Values indicate number of alleles or genotypes (%). *P* values test departure from the Hardy–Weinberg equilibrium.

arterial pressure and smoking were correlates of one or more retinal phenotypes. For consistency, we adjusted all analyses for sex (if appropriate), age, mean arterial pressure, and smoking.

Association between microvascular phenotypes and genetic polymorphisms

The genotypes and allele frequencies appear in Table 4. The genotype frequency of *AT2R* (*1675G/A*) in women complied with the Hardy–Weinberg equilibrium ($P = 0.99$). While accounting for relatedness and with adjustments applied for sex, age, mean arterial pressure, and smoking, CRAE was significantly higher in hemizygous and homozygous carriers of the *AT2R A* allele than in their *G* allele counterparts (Table 4) among women and men combined (+4.49 μm; $P = 0.014$) and among men (+4.91 μm; $P = 0.032$). In women, *AA* homozygotes had a slightly higher CRAE than *G* allele carriers, but this trend (+3.41 μm; $P = 0.14$) did not reach significance (Table 5). In line with CRAE findings, AVR was significantly higher in hemizygous and homozygous carriers of the *AT2R A* allele than in their *G* allele counterparts (Table 5).

Of our study participants, 15.0% were taking diuretics, 17.3% β-blockers, 6.5% vasodilators, and 10.6% inhibitors of renin–angiotensin–aldosterone system. Sensitivity analyses in which we additionally adjusted for antihypertensive drug treatment using design variables for four major drug classes were confirmatory.

DISCUSSION

In the present study, using a candidate gene approach, we focused on the retinal vasculature in relation to the *AT2R*

Table 5 | Association of retinal vascular phenotypes with gene polymorphisms

Genes	Mean ± s.e.		Difference (95% CI)	*P*
AT2R (women)	*AA* (*n* = 50)	*AG*+*GG* (*n* = 125)		
CRAE	159.1 ± 2.0	155.7 ± 1.4	3.41 (−1.12 to 7.95)	0.14
CRVE	217.1 ± 2.9	219.6 ± 2.0	−2.48 (−9.03 to 4.07)	0.45
AVR	0.738 ± 0.010	0.712 ± 0.007	0.025 (0.004–0.047)	0.021
AT2R (men)	*A* (*n* = 86)	*G* (*n* = 79)		
CRAE	153.1 ± 2.0	148.2 ± 2.0	4.91 (0.43–9.38)	0.0320
CRVE	217.0 ± 2.7	216.7 ± 2.6	0.292 (−5.762 to 6.346)	0.92
AVR	0.712 ± 0.009	0.686 ± 0.009	0.027 (0.005–0.048)	0.017
AT2R (all)	*AA* or *A* (*n* = 136)	*GG* or *G* (*n* = 118)		
CRAE	155.6 ± 1.5	151.1 ± 1.5	4.49 (0.92–8.06)	0.0143
CRVE	217.0 ± 2.1	216.3 ± 2.1	0.638 (−4.178 to 5.454)	0.79
AVR	0.724 ± 0.007	0.700 ± 0.008	0.024 (0.006–0.041)	0.0082

The measurements accounted for family cluster and were adjusted for sex, age, mean arterial pressure, and current smoking.
AT2R, angiotension II type-2 receptor; AVR, arteriole-to-venule ratio; CI, confidence interval; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent.

1675G/A polymorphism. The retinal vessels as visualized by nonmydriatic retinal imaging are believed to reflect the systemic microcirculation and carry important prognostic information. To our knowledge no previous study addressed the association of this phenotype with genetic variation in the X-linked *AT2R* gene. The key finding of our study was that *AT2R AA* homozygotes and *A* hemizygotes compared with their *GG* and *G* counterparts had higher CRAE in both sexes combined and in men with a similar trend in women.

Taarnhøj *et al.*[13] and colleagues enrolled 55 monozygotic and 50 dizygotic same-sex healthy twin pairs, aged 20–46 years, in a study of the heritability of the retinal microcirculation. Heritability was 70% (95% CI: 54–80%) for CRAE and 83% (95% CI: 73–89%) for CRVE.[13] As in our current study, retinal artery diameter decreased with advancing age and higher arterial blood pressure. As reviewed in detail elsewhere,[14] all components of the renin–angiotensin system are expressed within the retina. Angiotensin II, the main effector peptide of the renin–angiotensin system causes vasoconstriction of the retinal arterioles,[15] capillaries,[16] and venules.[17] In addition, there is evidence that angiotensin II also regulates glial[18] and neuronal[19] function in the central nervous system, of which the retina is part. Most aforementioned effects of angiotensin II are mediated via AT1R. However, both AT1R and AT2R are expressed in the retina of rats[20] and humans.[21] Moreover, several genome-wide association studies[22–24] highlighted loci, which were significantly related to the arterial and venular diameters. Thus, evidence from heritability, genome-wide association studies and expression studies justifies our prespecified hypothesis and current analysis.

The *AT2R 1675G/A* polymorphism is likely to be functional. It is located in the short intron 1 of the *AT2R* gene within a sequence motif similar to the splice branch site consensus.[2] Warnecke *et al.*[2] and colleagues investigated 12 explanted human hearts from 2 *GG* homozygous women and 10 hemizygous men (7 *A* and 3 *G* allele carriers). Irrespective of the allele present, the *AT2R* splice pattern was similar with a relative abundance of transcript exon 1/2/3 compared with exon 1/3. However, in transfected human (HT1080 (fibrosarcoma)) and rat (PC12W (pheochromocytoma)) cell lines and in rat smooth muscle cells, the luciferase activity driven by the *G* allele construct was significantly higher than that expressed from the *A* allele. Warnecke's observations[2] indicated that individuals carrying the *G* allele may express higher levels of AT2R protein, but not mRNA. In contrast, Nishimura *et al.*[25] and colleagues identified in primary fibroblasts derived from men and in uterine tissue from homozygous women differences in the *AT2R* splice pattern. *A* allele carriers expressed only splice variant exon 1/2/3 at a high level, whereas *G* allele carriers expressed only splice variant exon 1/3 at a low level. Nishimura and colleagues suggested that the *G* allele might be associated with reduced AT2R transcription, although they did not present protein expression data in support of that contention. The apparent discrepancies between the aforementioned studies might be due to the use of different tissues and cell models. However, both studies[2,25] suggest that the *AT2R 1675G/A* polymorphism might lead to differences in AT2R expression, which might be important from a pathophysiological point of view.

Whether the lower diameter of the retinal arterioles in hemizygous or homozygous carriers of the *AT2R G* allele is due to structural or functional alterations remains to be elucidated. AT2R regulates cell growth.[26] It is expressed at low levels in normal tissues,[26] but upregulated in pathological states.[4,27] In the presence of hypertension or high-sodium intake, left ventricular mass as measured by echocardiography[6] or magnetic resonance imaging[28] was higher in *AT2R G* allele carriers. These observations might favor a structural explanation of our current findings, for instance by hyperplasia or hypertrophy of the vascular smooth muscle cells. On the other hand, activation of the AT2R receptor might attenuate or antagonize AT1R-mediated physiologic effects. In favor of a functional explanation are experiments

showing an increased retinal blood flow in response to flicker light stimulation, which might be due to an AT2R-mediated nitric oxide-dependent mechanism.[29–31]

The present study must be interpreted within the context of its limitations. First, the sample size was small and the participants had a wide range of age and blood pressure. Second, the retinal photographs were taken independent of the cardiac cycle and effects of pulsatility on vessel width cannot be ruled out.[11] Third, we did not adjust for multiple testing. Adjustment for multiple comparisons is usually recommended to avoid rejecting null hypotheses too readily.[32] In the present study the correlation between the three phenotypes of interest (CRAE, CRVE, and AVR) were ≥0.49, In such case, multiple testing is not indicated, because each new test does not provide a completely independent opportunity for a type I error.[33,34] Moreover, our main findings were hypothesis driven and focused on a single polymorphism in the *AT2R* gene.

In conclusion, retinal microvascular phenotypes might be associated with the *AT2R 1675G/A* polymorphism. However, these observations need confirmation in a larger population sample and different cohorts.

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